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Positivity of both plasma Epstein–Barr virus DNA and serum Epstein–Barr virus capsid specific immunoglobulin A is a better prognostic biomarker for nasopharyngeal carcinoma

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ABSTRACT

Background: Positivity of plasma Epstein–Barr virus (EBV)-DNA or serum virus capsid antigen-specific IgA (VCA-IgA) is a biomarker for the prognosis of nasopharyngeal carcinoma (NPC). The objective of this study was to determine the value of positivity for plasma EBV-DNA and/or VCA-IgA in predicting the survival of patients with NPC.

Methods: Plasma EBV-DNA and serum VCA-IgA in 506 NPC patients in this retrospective study were detected by quantitative real time polymerase chain reaction and enzyme-linked immunoabsorbent assay, respectively. The value of positivity for EBV-DNA and/or VCA-IgA in predicting the survival of patients with NPC was analyzed.

Results: Patients with positivity for both EBV-DNA and VCA-IgA had significantly shorter periods of relapse free survival (RFS) and overall survival (OS) than those with positive single measure or negative for both measures, and patients with positive single measure had significantly shorter periods of RFS and OS than those with negative for both. Multivariate analysis indicated that the positivity for EBV-DNA and/or VCA-IgA were significant risk factors for shorter periods of RFS and OS.

Conclusion: These data indicated that positivity for both EBV-DNA and VCA-IgA was a better biomarker for the prognosis of patients with NPC. Our findings may provide new references for clinical practice.

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1. Introduction

Nasopharyngeal carcinoma (NPC) is a unique type of cancers in the head and neck because of its epidemiology, histopathology, clinical characteristics, therapeutic strategies, and treatment outcomes. Currently, the incidence of NPC in the Southern China and Asia is especially high, nearly 30 per 100,000 [1]. While radiotherapy is considered as the primary strategy for treatment of NPC patients without distant metastasis, [2] chemoradiotherapy is a better therapeutic modality for patients with advanced disease [3]. Although these therapeutic strategies have significantly improved the survival rate and life quality of patients with NPC, the 5-year survival probability is still low in NPC patients due to the disease recurrence and distant metastasis [4]. Hence,

discovery of risk factors for the recurrence and metastasis will be of great significance in the prognosis and management of NPC patients.

It is well known that Epstein–Barr virus (EBV) infection is associated with NPC. Indeed, the EBV DNA can be detected in NPC cells [5–8]. Previous studies have shown that plasma EBV-DNA [9–15] and serum EBV capsid antigen-specific IgA (VCA-IgA) [16–21] are detected in patients with NPC and have been used as biomarkers for screening, diagnosis, monitoring, and prognosis of NPC. Indeed, the levels of plasma EBV-DNA loads in newly diagnosed NPC patients are significantly correlated with tumor volume [22,23], tumor clearance [24,25], poor responses to chemoradiation [26], and tumor recurrence [27–29]. Similarly, seropositive VCA-IgA is associated with increased risk for NPC [30,31] and NPC patients with higher levels of VCA-IgA have a worse prognosis [18–21]. It has been thought that plasma EBV-DNA can be derived from apoptotic tumor fragments and VCA-IgA reflects mucosal immune responses to EBV replication. They are different biological events, associated with the development of NPC. However, some patients are only positive for EBV-DNA or VCA-IgA and the value of plasma EBV-DNA or VCA-IgA for the prognosis is inconsistent [9,20,32–35]. Currently, there is no

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information on the value of positivity for both plasma EBV-DNA and VCA-IgA in the prognosis of NPC. Indeed, it is unclear whether combined plasma EBV-DNA and serum VCA-IgA can be a better marker for the prognosis of NPC patients. Given that identification of risk for disease recurrence and poor survival in NPC patients is crucial for early intervention, addressing these questions will benefit patients with NPC. It is urgent to determine the prognostic values of positive plasma EBV-DNA and/or seropositive VCA-IgA in NPC patients.

The objective of this study was to determine whether positivity for plasma EBV-DNA and/or VCA-IgA could be a better biomarker for predicting the poor survival of patients with NPC. A total of 506 patients with NPC were selected for this retrospective study. The presence of plasma EBV-DNA and serum VCA-IgA in these patients was determined before treatment. Subsequently, these patients were stratified and the association of positivity for both or single measure with the disease relapse, mortality, relapse free survival (RFS) and overall survival (OS) of NPC patients was analyzed. We found that the positivity for both measures was associated with significantly higher rates of mortality and disease relapse, as well as shorter periods of RFS and OS than that in those with positivity for single measure or negativity for both measures in this population. We discussed the implications of our findings.

2. Materials and methods

2.1. Patients and clinical specimens

A total of 506 patients with biopsy-proven, newly diagnosed NPC were selected from Department of Otolaryngology—Head and Neck Surgery of Nanfang Hospital in Southern Medical University from January 2006 to April 2013. Individual patients with NPC were diagnosed, based on the pathological examination of biopsied tissues. The pathogenic stages of individual patients were according to the 2002 American Joint Committee on Cancer TNM staging system. Individual patients with another type of tumor were excluded.

Venous blood samples were collected from individual patients before treatment. Their plasma and serum samples were prepared. The tests of plasma EBV-DNA and VCA-IgA were performed by the Department of Laboratory of Nanfang Hospital. This study was approved by the Institutional Review Board of our hospital, and written informed consent about analysis of their clinical parameters was obtained from individual patients before treatment.

2.2. Clinical management

All patients received conventional two-dimensional radiotherapy uniformly administered to the primary tumor and neck region with a total dose of 66 to 70 Gy over a 6–8-week period. There were 410 (81.03%) patients with advanced disease (T₃–T₄ or N₂–N₃) who also received induction chemotherapy prior to radiation or adjuvant chemotherapy after radiation.

After the last chemoradiotherapy, all patients were examined at 3, 6, and 12 months in the first year, every 6 months during the second and third years, and yearly thereafter. The recurrence of NPC was evaluated by clinical physical examination, magnetic resonance imaging (MRI) and/or computed tomography (CT) scans from the skull base to the whole neck, chest radiography, whole-body bone scan, abdominal sonography, fiber nasopharyngoscopy and biopsied pathologic verification if necessary. PET-CT examination was used to ensure distant metastasis.

2.3. Quantification of plasma EBV-DNA

The total plasma DNA was extracted and the contents of plasma EBV-DNA were quantified by quantitative real time polymerase chain reaction (qRT-PCR) using an EBV RT-PCR kit (Liferiver, Shanghai,

China), according to the manufacturers' instruction. The cut-off value for a positive plasma EBV DNA load is >0 copy/ml.

2.4. Serologic analysis of VCA-IgA

The concentrations of serum VCA-IgA were determined by enzyme-linked immunoabsorbent assay (ELISA) using a specific kit (Beier, Beijing, China), according to the manufacturers' instruction. The cut-off value for positive EBV VCA-IgA is optical density (OD) of >1.

2.5. Statistical analyses

Data shown are the case numbers and the median and range. Patients were stratified, according to individual measures and the relapse and mortality rates of each group of patients were analyzed by the chi-square test. The RFS was calculated from the first day of induction chemotherapy to the date of either disease recurrence or the last follow-up visit. The OS was calculated from the first day of induction chemotherapy to the date of either death or the last follow-up visit. The periods of RFS and OS among the different groups of patients were evaluated by Kaplan–Meier method and analyzed by the log-rank test. The potential risk of age, gender, tumor classification, lymph node status, metastasis status and status of plasma EBV-DNA and VCA-IgA for the poor survival of NPC patients was analyzed by hazard ratio (HR), 95% confidence interval (CI) and the Wald test using multivariate Cox proportional hazard model. All statistical analyses were performed by SPSS (version 19.0 for window; Statistical Product and Service Solutions; IBM). A two-tailed *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Patients' characteristics

To determine the potential importance of plasma EBV-DNA and serum VCA-IgA positivity in the prognosis of NPC, 506 patients with newly diagnosed NPC were selected. The demographic and clinical characteristics are shown in Table 1. There were 69.56% of patients with positive plasma EBV-DNA and 45.85% of patients with positive serum VCA-IgA in this population. Stratification of patients, according to the positivity of EBV-DNA and VCA-IgA indicated that there was a significant difference in the percentages of NPC patients with positivity for EBV-DNA alone, for VCA-IgA alone, for both EBV-DNA and VCA-IgA and negativity for both measures (*P* = 0.008) (Table 2). A total of 175 (34.58%) patients were positive for both plasma EBV-DNA and serum VCA-IgA, 177 (34.98%) patients with positive plasma EBV-DNA alone, 57 (11.27%) patients with positive serum VCA-IgA alone and 97 (19.17%) patients with negative plasma EBV-DNA and serum VCA-IgA. There was no significant difference in the distribution of age, gender, and pathologic classification among these four groups of patients. However, there was a significant difference in the distribution of tumor classification, lymph node status, metastasis status and overall stage among these four groups of patients. During the median observation of 37 (range 5 to 75) months, there were 116 (22.92%) patients with NPC recurrence and 96 (18.97%) patients died. During the same period, there were 23 patients with NPC relapse in nasopharynx and 8 in neck, while 68 patients developed distant metastasis, 10 with nasopharynx relapse and distant metastasis, and 7 with neck relapse and distant metastasis.

3.2. Survival analysis

Stratification analyses indicated that there was a significant difference in the rates of NPC relapse and mortality among these four groups of patients (*P* < 0.001 for both) (Fig. 1A,C). As a result, there was a significant difference in the periods of RFS and OS among these four groups of patients (*P* < 0.001 for both) (Fig. 1B,D). Further analyses revealed that

Table 1
The demographic and clinical characteristics of NPC patients.

Characteristic	All	EBV-DNA +/VCA-IgA +	EBV-DNA +/VCA-IgA –	EBV-DNA –/VCA-IgA +	EBV-DNA –/VCA-IgA –	P values
Case no.	506	175 (34.58)	177 (34.98)	57 (11.27)	97 (19.17)	$P^a < 0.001$
Age (yrs)						$P^b = 0.478$
Median	44	47	45	48	46	
Range	17–80	17–80	18–77	17–75	19–70	
Gender						$P^a = 0.153$
Male	380 (75.10)	142 (81.14)	128 (72.32)	41 (71.93)	69 (71.13)	
Female	126 (24.90)	33 (18.86)	49 (27.68)	16 (28.07)	28 (28.87)	
WHO pathologic classification						$P^b = 0.362$
Type I	9 (1.78)	2 (1.14)	1 (0.56)	2 (3.51)	4 (4.12)	
Type II	22 (4.35)	9 (5.14)	8 (4.52)	2 (3.51)	3 (3.09)	
Type III	475 (93.87)	164 (93.72)	168 (94.92)	53 (92.98)	90 (92.79)	
Tumor classification						$P^b < 0.001$
T1	84 (16.60)	25 (14.29)	20 (11.30)	11 (19.30)	28 (28.87)	
T2	110 (21.74)	31 (17.71)	37 (20.90)	17 (29.82)	25 (25.77)	
T3	153 (30.24)	63 (36.00)	53 (29.95)	16 (28.07)	21 (21.65)	
T4	159 (31.42)	56 (32.00)	67 (37.85)	13 (22.81)	23 (23.71)	
Lymph node status						$P^b < 0.001$
N0	55 (10.87)	10 (5.71)	13 (7.35)	14 (24.56)	18 (18.56)	
N1	156 (30.83)	33 (18.86)	61 (34.46)	18 (31.58)	44 (45.36)	
N2	206 (40.71)	86 (49.14)	73 (41.24)	21 (36.84)	26 (26.80)	
N3	89 (17.59)	46 (26.29)	30 (16.95)	4 (7.02)	9 (9.28)	
Metastasis status						$P^a = 0.004$
M0	478 (94.47)	162 (92.57)	162 (91.53)	57 (100)	97 (100)	
M1	28 (5.53)	13 (7.43)	15 (8.47)	0 (0)	0 (0)	
Overall stage						$P^b < 0.001$
I	14 (2.77)	1 (0.57)	2 (1.13)	3 (5.26)	8 (8.25)	
II	82 (16.21)	17 (9.71)	20 (11.30)	18 (31.58)	27 (27.84)	
III	177 (34.98)	63 (36.00)	62 (35.03)	20 (35.09)	32 (32.99)	
IV	233 (46.04)	94 (53.72)	93 (52.54)	16 (28.07)	30 (30.92)	

Data shown are the real case numbers (%), unless specified. P^a values were determined using the chi-square test; P^b values were determined using the k independent samples test.

the periods of RFS and OS in patients with positivity for both EBV-DNA and VCA-IgA were significantly shorter than that of patients with positivity for EBV-DNA alone, with positivity for VCA-IgA alone and those with negativity for both EBV-DNA and VCA-IgA (Supplementary Table 1). The periods of RFS and OS in patients with positive plasma EBV-DNA alone were significantly shorter than that of patients with negativity for both ($P = 0.006$ for both). However, there was no significant difference in the periods of RFS and OS between the patients with positive EBV-DNA alone and with seropositive VCA-IgA alone. Similarly, there was no significant difference in the periods of RFS and OS between the patients with seropositive VCA-IgA alone and those with negativity for both EBV-DNA and VCA-IgA. Accordingly, the risk for NPC relapse and mortality in patients with positivity for both EBV-DNA and VCA-IgA was higher than that of those with positivity for EBV-DNA alone, which was also higher than that of patients with positivity for VCA-IgA alone and those with negativity for both measures.

Given small sample size and no significant difference in the periods of RFS and OS between the patients with single positivity of either EBV-DNA or VCA-IgA, we further combined these two groups of patients to analyze the relapse and mortality rates and the periods of RFS and OS among these three groups of patients. First, there was a significant difference in the relapse and mortality rates among these three groups of patients ($P < 0.001$ for both) (Fig. 2A,C). Similarly, there was a significant difference in the periods of RFS and OS among these three groups of patients ($P < 0.001$ for both) (Fig. 2B,D). Further analyses revealed that the periods of RFS and OS in the patients with positivity for both

EBV-DNA and VCA-IgA were significantly shorter than that of patients with single positivity of EBV-DNA or VCA-IgA ($P = 0.003$ for RFS; $P = 0.004$ for OS) (Supplementary Table 2) and those with negativity for both ($P < 0.001$ for both). Interestingly, the periods of RFS and OS in the patients with single positivity of EBV-DNA or VCA-IgA were significantly shorter than that of those with negativity for both measures ($P = 0.011$ for RFS; $P = 0.010$ for OS). These data further indicated that the risk for relapse and mortality in NPC patients with positivity for both EBV-DNA and VCA-IgA was higher than that of patients with single positive EBV-DNA or VCA-IgA, which also was higher than that of those with negativity for both measures.

3.3. Cox multivariate analysis

Next, we performed the multivariate analysis using the multivariate Cox proportional hazard model. The lymph node metastasis was a significant risk factor for the shorter RFS periods in NPC patients (HR: 1.555; 95% CI: 1.248–1.937; $P < 0.001$) (Table 3). Similarly, the older age (HR: 1.976; 95% CI: 1.268–3.079; $P = 0.003$), higher tumor classification (HR: 1.378; 95% CI: 1.118–1.698; $P = 0.003$), lymph node metastasis (HR: 1.639; 95% CI: 1.261–2.131; $P < 0.001$) and metastasis (HR: 4.842; 95% CI: 2.721–8.618; $P < 0.001$) were significant risk factors for shorter periods of OS in NPC patients. The positivity for EBV-DNA and/or VCA-IgA were identified as significant risk factors for shorter periods of RFS ($P = 0.001$) and OS ($P = 0.002$). In comparison with that in the patients with negativity for both EBV-DNA and VCA-IgA with a designated HR of 1, the positivity for both EBV-DNA and VCA-IgA (HR: 3.644; 95% CI: 1.717–7.732; $P = 0.001$), single positive EBV-DNA or VCA-IgA (HR: 2.400; 95% CI: 1.131–5.096; $P = 0.023$), were significant risk factors for the shorter RFS periods. Furthermore, the positivity for both EBV-DNA and VCA-IgA (HR: 3.972; 95% CI: 1.554–10.153; $P = 0.004$), but not positive single EBV-DNA or VCA-IgA (HR: 2.226; 95% CI: 0.863–5.739; $P = 0.098$), was a significant risk factor for the shorter OS periods in NPC patients.

Table 2
The positivity of plasma EBV-DNA and serum VCA-IgA.

		VCA-IgA	
		Positive	Negative
EBV-DNA	Positive	175 (34.58%)	177 (34.98%)
	Negative	57 (11.27%)	97 (19.17%)

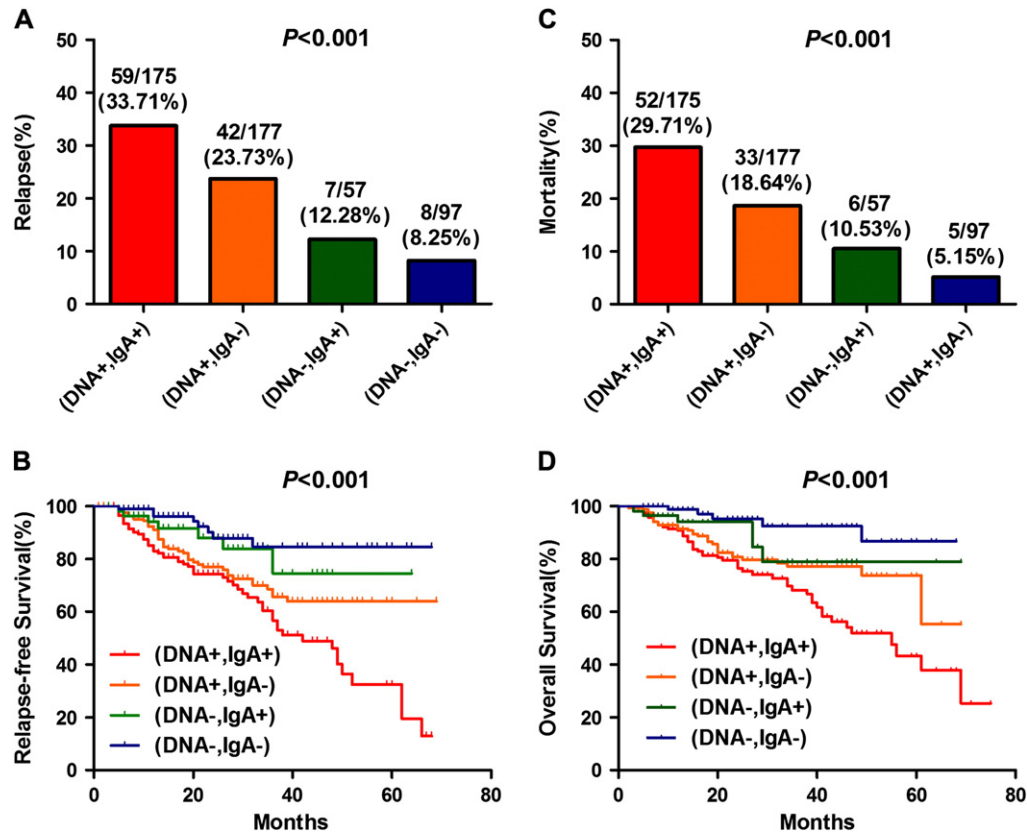


Fig. 1. Stratification analyses of the survival of NPC patients. All of the NPC patients were stratified, according to the detection of EBV-DNA and VCA-IgA and their NPC relapse and mortality were calculated. Subsequently, the periods of RFS and OS of individual groups of patients were estimated by the Kaplan–Meier method and analyzed by the Log rank test. (A) The percentages of relapse. (B) The percentages of RFS in the different groups of NPC patients throughout the observation period. (C) The percentages of mortality. (D) The percentages of OS in the different groups of patients throughout the observation period.

4. Discussion

Previous studies have shown that positive plasma EBV-DNA or serum VCA-IgA is prognostic factor for the relapse and survival of NPC patients [12–15,18–21,27]. However, these markers do not always appear simultaneously in NPC patients [9,20,32–34]. In this retrospective study, we compared the risks of positivity for EBV-DNA and/or VCA-IgA in predicting the relapse of NPC and the survival of 506 NPC patients. We found 69.56% of patients with positive plasma EBV-DNA and 45.85% of patients with positive serum VCA-IgA in this population. According to the positive detection of EBV-DNA and VCA-IgA, there were four groups of patients with positivity for both EBV-DNA and VCA-IgA, EBV-DNA alone, VCA-IgA alone, and negative for both measures. The percentages of patients with both EBV-DNA and VCA-IgA or EBV-DNA alone were significantly higher than that of those with positive VCA-IgA alone or negative both measures. These data suggest that majority of NPC patients had EBV infection and some patients developed IgA responses to VCA in this population. These data also support the notion that EBV infection is associated with the development of NPC [5–8].

EBV-DNA and VCA-IgA have been routinely tested in NPC patients and positive plasma EBV-DNA or serum VCA-IgA is a prognostic factor for the relapse and survival of NPC patients. In this study, the positivity rates in detection of EBV-DNA in NPC patients were significantly higher than that of VCA-IgA, consistent with previous reports [32–34]. However, there were still more than 30% of NPC patients who were negative for EBV-DNA. If combined with VCA-IgA, the detection sensitivity is 80.83%, better than EBV-DNA alone. The combination of EBV-DNA and VCA-IgA may be more suitable to diagnose NPC. Baizig [20] also reported that there was no significant correlation between the levels of plasma EBV-DNA and serum VCA-IgA in NPC cases. Twu [35] reported that EBV-DNA is superior to VCA-IgA in prognostic prediction of NPC. However,

we found that patients with positivity for both measures had a worse prognosis than those with positivity for EBV-DNA alone (Fig. 1), and VCA-IgA can also predict the prognostic of NPC in our study (Supplementary Fig. 1). Therefore, positivity for both EBV-DNA and VCA-IgA is a better marker for the prognosis of NPC patients.

Physicians are commonly confused with biomarkers for the prognosis of NPC patients because of the inconsistency of positivity for EBV-DNA and VCA-IgA. We compared the periods of RFS and OS among these 506 patients. We found that patients with positivity of both measures had the worst RFS and OS while patients with negativity for both measures had the longest periods of RFS and OS among these patients. Furthermore, patients either positive plasma EBV-DNA or serum VCA-IgA had significantly shorter survival periods than those with negativity for both measures. Further multivariate analyses revealed that positivity for both plasma EBV-DNA and serum VCA-IgA was a risk factor for the recurrence and mortality of NPC patients. Apparently, the risk for the recurrence and mortality of NPC may range from patients with positivity for both measures, positivity for either single measure to negativity for both measures. Indeed, a recent study has shown that the contents of plasma EBV-DNA, together with a new clinical typing system, can stratify the NPC patients into four distinct risk groups and are valuable for the prognosis of NPC [36]. Hence, simultaneous tests of plasma EBV-DNA and serum VCA-IgA are valuable to identify the NPC patients at high risk for the recurrence and mortality. Accordingly, we should closely follow up those patients with positivity for both measures to early detect and treat the recurrence and metastasis of NPC. To the best of our knowledge, this was the first study on the value of combination of EBV-DNA and VCA-IgA in the prognosis of NPC patients. Our findings may provide new references for clinical practice. We are interested in further investigating the clinical significance of early aggressive treatment of patients with positivity for both measures.

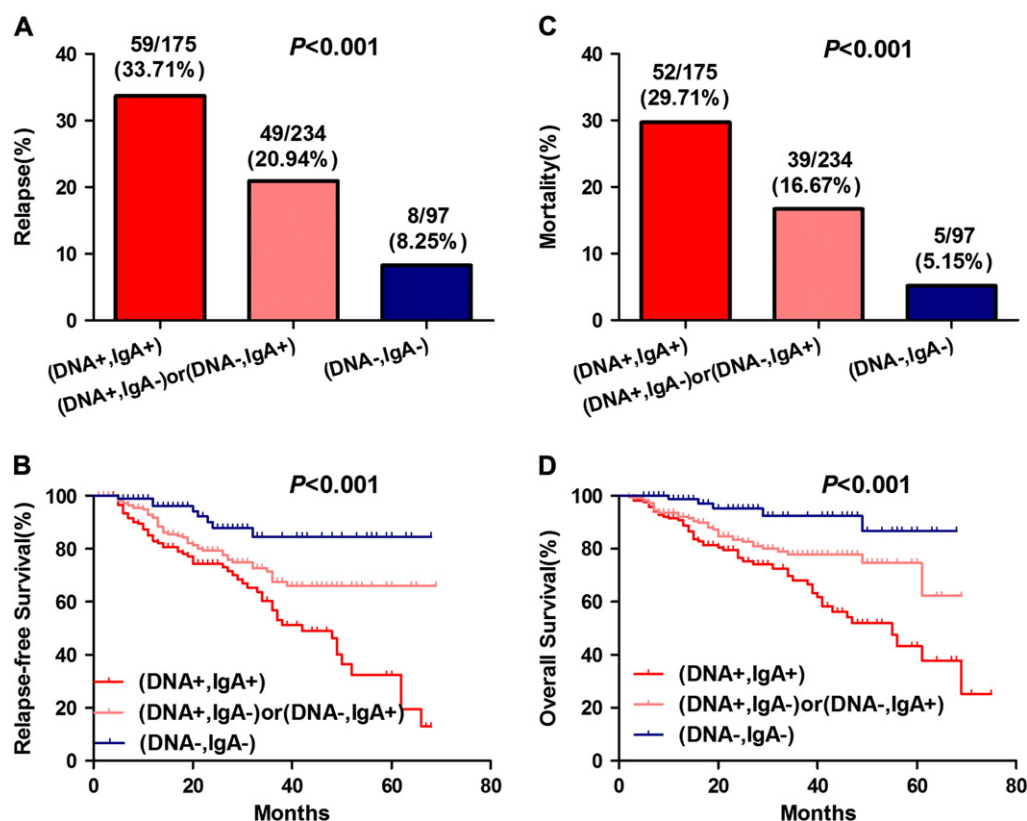


Fig. 2. Stratification analyses of the survival of NPC patients. All of the NPC patients were stratified, according to the positivity for both EBV-DNA and VCA-IgA, for single positive measure or negative for both measures, and their NPC relapse and mortality were calculated. Subsequently, the periods of RFS and OS of individual groups of patients were estimated by the Kaplan-Meier method and analyzed by the Log rank test. (A) The percentages of relapse. (B) The percentages of RFS in the different groups of NPC patients throughout the observation period. (C) The percentages of mortality. (D) The percentages of OS in the different groups of patients throughout the observation period.

We recognized that our study had limitations, including a retrospective study with limited sample size, most patients with pathologic type III of NPC and small sample size for the patients with positively serum VCA-IgA alone. In this case, we had no strong data to distinguish the risk between the patients with EBV-DNA alone and VCA-IgA alone. Therefore, further prospective studies in a bigger population to validate these findings and dissect the risk for the recurrence and mortality between patients with positivity for either EBV-DNA or VCA-IgA as well as the levels of plasma EBV-DNA and serum VCA-IgA in the prognosis of NPC are warranted.

5. Conclusions

In summary, our data indicated that positivity for both plasma EBV-DNA and serum IgA had a significantly higher risk for the recurrence and mortality in patients with NPC. Simultaneous tests of both EBV-DNA and VCA-IgA may be valuable for the prognosis of NPC in the clinic.

Therefore, our findings may provide a new reference for management of patients with NPC in the clinic.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbacli.2014.10.003>.

Conflict of interest disclosures

The authors made no disclosures.

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Table 3
Relapse-free and overall survival analyses using a multivariate Cox proportional hazards model.

Parameter	RFS		OS	
	HR (95% CI)	<i>P</i> ^a	HR (95% CI)	<i>P</i> ^a
Age: ≥44 y vs <44 y	1.195 (0.819, 1.743)	0.357	1.976 (1.268, 3.079)	0.003
Sex: men vs women	0.665 (0.411, 1.074)	0.095	0.893 (0.543, 1.469)	0.656
Tumor classification: T1, T2, T3, and T4	1.152 (0.962, 1.379)	0.125	1.378 (1.118, 1.698)	0.003
Lymph node status: N0, N1, N2, and N3	1.555 (1.248, 1.937)	<0.001	1.639 (1.261, 2.131)	<0.001
Metastasis status: 1 vs 0	0.323 (0.078, 1.332)	0.118	4.842 (2.721, 8.618)	<0.001
Markers: (DNA+, IgA+), (DNA+, IgA-) or (DNA-, IgA+), (DNA-, IgA-)*		0.001		0.002
(DNA+, IgA+)	3.644 (1.717, 7.732)	0.001	3.972 (1.554, 10.153)	0.004
(DNA+, IgA-) or (DNA-, IgA+)	2.400 (1.131, 5.096)	0.023	2.226 (0.863, 5.739)	0.098

^a *P* values were determined using the Wald test. *(DNA-, IgA-) was defined as reference group.

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